

then it would appear that one of the effects of the introduction of oxygen is to diminish the part played by water in the reactions.

In conclusion we wish to express our thanks to Prof. F. Gowland Hopkins, in whose laboratory this work was done.

Anthocyanins and Anthocyanidins. Part IV.—*Observations on :*
(a) *Anthocyan Colours in Flowers, and* (b) *the Formation of*
Anthocyanins in Plants.

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(Communicated by Prof. F. Keeble, F.R.S.—Received February 2, 1921.)

In a recent paper, Shibata, Shibata and Kasiwagi,* dealing with the question of the colours observed in flowers, and the condition of the anthocyan pigments as they exist in plants, arrived at conclusions that differ considerably from those of Willstätter and of Everest. In view of this, and of the fact that Shibata, Shibata and Kasiwagi's paper has passed into reviews, the present authors feel that the publication of some observations that they have made, both before the publication of the paper referred to, and since receiving it, as confirmation of the present authors' results and extension of the field, will not be out of place.

Results of experiments upon the formation of anthocyan pigments are also described, and important conclusions drawn from them.

The Constitution of the Blue Anthocyan Pigments in Flowers.

Willstätter and Everest† as the result of their examination of the pigments of the cornflower and of preliminary investigations upon other flowers, concluded that the blue colour in the cornflower was due to an alkali, or alkali-earth, salt of a phenolic substance which was violet in the free state, and which was also capable of forming red oxonium salts with acids. This simple explanation of the main colour changes being due to changes in the condition of the cell sap in the plants concerned, has been elaborated by Willstätter and Mallison‡ to show how such a supposition, coupled with the

* 'J. Amer. Chem. Soc.,' vol. 41, p. 208 (1919).

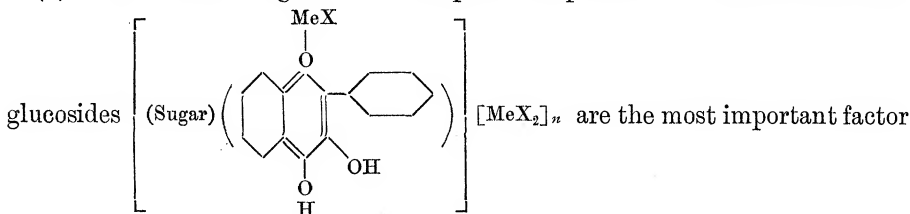
† 'Ann.,' vol. 401, p. 189 (1913).

‡ 'Ann.,' vol. 408, p. 147 (1915).

effects obtained by the presence of other plant colouring matters (flavonols, carotinoids, etc.) and of varying concentration of colour in the cell sap, is capable of explaining all the lovely variations seen in plant life.

Shibata, Shibata and Kasiwagi challenge the evidence of the existence of the anthocyan pigments in the three forms suggested by Willstätter and Everest, and to replace the conclusions of Willstätter and Everest, and Willstätter and Mallison, Shibata, Shibata and Kasiwagi put forward the following as an explanation of the various flower colours:—

(1) "The metal organic or complex compounds of reduced flavonal



in the production of flower colours."

(2) "The 'blue' anthocyanins are the complex compounds of reduced flavonol glucosides, which possess several hydroxyl groups belonging to the flavonol nucleus besides those of sugar molecules, and the metal with which they are coordinated is probably calcium or magnesium, for the salts of these metals are always present in the plant cells."

(3) "The 'violet,' 'violet red' or 'red,' pigments are either the analogous metallic complex compounds of flavonol glucosides, which contain fewer of the auxochromic hydroxyl groups, or a mixture of the blue pigments and their decomposition products by excess of acids, *i.e.*, the red oxonium salts of R. Willstätter."

They then add, "Our conception of the colour variation of the anthocyanins is, we believe, free from the objections raised in the preceding pages, and moreover, the blue pigments are accessible, while the phenolates and inner salts are wanting an experimental support."

Before passing to observations concerning these new suggestions, it is advantageous to consider carefully the experimental evidence that is available in support of them, as compared with that upon which the earlier conclusions of Willstätter and Everest were founded and developed.

Willstätter and Everest's conclusions are supported by considerably more experimental evidence than that referred to by Shibata, Shibata and Kasiwagi, and it would appear that these authors have overlooked some of the later work of Willstätter and his collaborators.

Willstätter and Everest (*loc. cit.*) produced evidence that proved the formation of oxonium salts in red anthocyanins, and made it highly probable that

the blue pigment of the cornflower was the potassium or calcium salt of cyanin. Willstätter and Mieg proved that the violet flowers of wild larkspur contained the neutral colour base of delphinin,* whilst Willstätter and Bolton† showed that in the scarlet pelargonium the pigment pelargonin was accompanied by a considerable amount of tartaric acid, and that it could form stable salts (oxonium) with such weak acids as acetic acid. The further work of Willstätter and his collaborators has resulted in the isolation of the colour bases of the pigments: pelargonin, cyanidin and delphinin, and also of the colourless pseudo-bases of pelargonidin, cyanidin and delphinidin.

In support of their theory of complex salts, and their explanation of the colours in flowers based thereon, Shibata, Shibata and Kasiwagi describe the preparation of various blue and blue-green compounds and give analyses of them.

Exhaustive and careful examination of the data given by them has brought the present authors to the conclusion that much of the evidence they set out is of an unsatisfactory character, and that their conclusions concerning their experimental results are misleading.

The present authors are convinced that very valuable results may often be obtained by the careful examination of plant extracts or impure pigments, but only if and when due allowance is made for the fact that impurities are present and for the nature of the impurities. In dealing with plant pigments, such allowances can only be made when the investigator concerned has himself made a careful study of the pure pigments and has considerable first-hand knowledge of these bodies. It is often the case that access to even the smallest amount of a pure pigment will save endless incorrect deduction from experimental results. It appears to the present authors that sufficient weight has not been attached to this by Shibata, Shibata and Kasiwagi.

Not only is this the case, but in places their arguments are inconsistent with statements made in other parts of their paper.

Willstätter, and also Everest, have found, as the result of quantitative work, that under conditions such as Shibata, Shibata and Kasiwagi described for the isolation of their green and blue pigments, the flavonol used is never quantitatively converted into the corresponding anthocyan. Furthermore, it is well known that the addition of metallic acetates in alcoholic solution to flavonol derivatives such as myricetin gives rise to the formation of metal phenolic salts of the flavonols. Hence, when they precipitated their green pigments, it follows that they obtained, not as they imagined, an anthocyan

* 'Ann.,' vol. 408, p. 67 (1915).

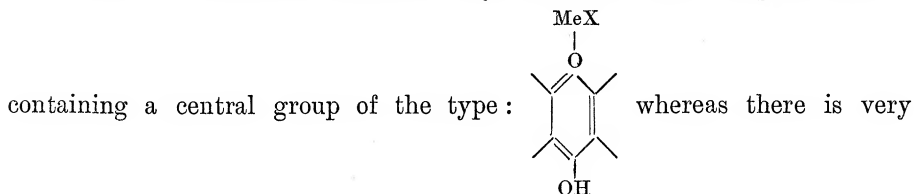
† 'Ann.,' vol. 408, p. 53 (1915).

salt, but a mixture of magnesium salts of the anthocyan and flavonol. Not only so, but magnesium acetate is appreciably soluble in alcohol, and is precipitated from its solution by means of ether. Their extraction of the precipitate with ether—to remove myricetin—would be of no avail, as the myricetin would be present, not free, but combined with the metal. Exactly similar results have been obtained by the present authors by mixing a trace of anthocyan with a flavonol in alcoholic solution, adding magnesium acetate, and precipitating and washing with ether. The green and greenish-blue pigments of Shibata, Shibata and Kasiwagi can be matched in this way with ease; they do not represent pure compounds, and Shibata, Shibata and Kasiwagi's analytical figures are of no value.

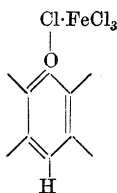
An important case of inconsistency arises in connection with the statements concerning their complex compounds. They argue that the blue anthocyan colours are complex metallic salts, and describe, as mentioned above, the preparation of what they consider to be these complex salts, but, on p. 211, they state that the products which they prepared "are soluble in water as well as in alcohol," and, on pp. 217, 218, say of their green pigments that they are soluble in alcohol as well as water. Despite this, they say on p. 213 that the "sparing solubility of many blue or bluish-violet anthocyanins in alcohol is quite consistent with the magnitude of the complex molecules." This latter statement would indicate that their compounds were not the same as the natural pigments.

The brownish red crystalline powder obtained by them (p. 218) was undoubtedly a mixture of flavonol and anthocyan, for the green or blue substances from which it was prepared must have contained the magnesium salt of myricetin.

It should further be noted that they formulate their complex salts as



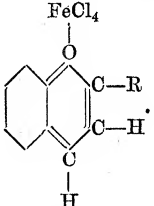
considerable chemical evidence (see below) that at least in the case of the salts with ferric chloride, the pyrylium ring is correctly represented thus :



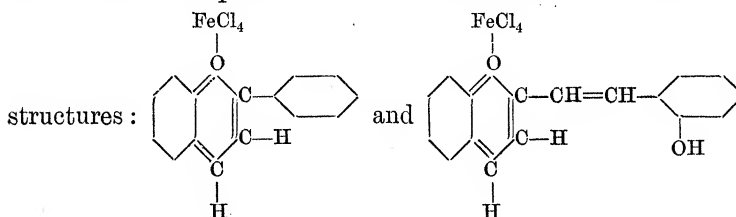
Comparison of the Properties of various Salts and simple Phenolates of Anthocyanins and related Pyrylium Derivatives.

From the authors' own observations and from previous work of other investigators, it is now apparent that two distinct classes of metallic salts of anthocyan pigments (or related hydroxylated pyrylium compounds) exist.

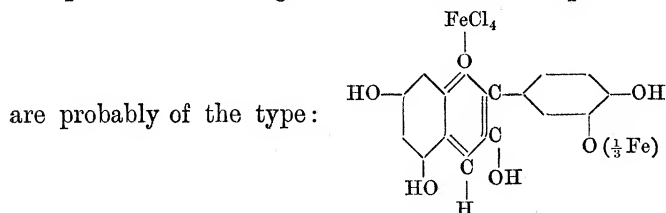
The work of others has shown that in numerous pyrylium compounds (oxonium salts) the addition of ferric chloride gives rise to iron compounds

which can be represented by simple formulæ of the type:  They

appear to be additive compounds of the iron salt and the oxonium salt of the pyrylium derivative. In connection with other work, the present authors have isolated compounds in which the iron chloride-content agrees with the



When dilute solutions of the oxonium salts of many anthocyanins have small amounts of iron salts added to them, characteristic intensely coloured solutions result (blue in the case of ferric chloride to cyanin or violanin chloride) which are stable and show no tendency to decolorise on standing. The present authors agree that these are complex salts, but consider they



On the other hand, sodium, potassium, calcium, or magnesium salts of the anthocyanins, when in solutions of similar dilution, gradually decolorise on standing. The present authors are of the opinion that these are simple phenolates, and related to the alkali salts of the flavonols described by A. G. Perkin and others.

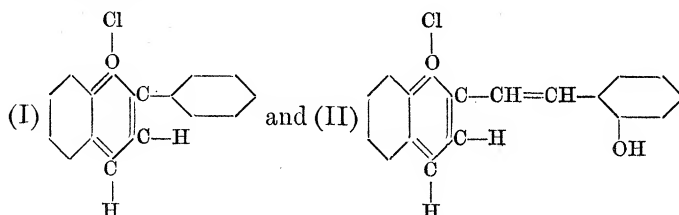
This indicates clearly a very marked line of differentiation between, the

action of iron salts and those of the alkali and alkali-earth metals on anthocyan pigments.

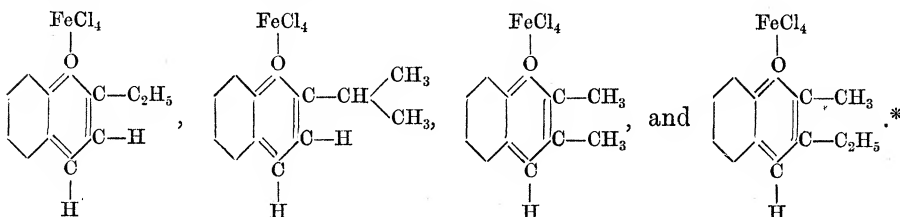
Confirmation and additional information concerning this difference has now been obtained by adding a trace of the salts of various metals to decolorised solutions of violanin or cyanin chlorine. In the case of iron, copper, and tin, although no immediate colour change occurred, a full intense blue gradually developed and was stable, whereas in the case of the salts of sodium, calcium, and magnesium, even on long standing, no return of colour at all occurred. (The presence of the anthocyan in the colourless form was proved in each case after the experiment by the addition of acid, whereby the oxonium salt was regenerated in full.)

In connexion with the complex iron salts, Willstätter's observations concerning the methylation of delphinidin upon the colour changes produced when the oxonium salts of delphinidin, or its various methyl ethers, are treated with iron chloride have made it abundantly clear that the final change from purple (or violet) to blue is, in these instances, dependent upon the reactivity of certain phenolic hydroxy groups.

The present authors have found that when iron chloride is added to the solutions of the compounds



there is no colour change beyond slight intensification, although definite iron compounds are formed, and the tendency to decolorise in dilute solution is very greatly reduced. This absence of marked colour change on passing from the oxonium chloride to the iron double salts has also been recorded in a number of cases, including the following:—



On the other hand, the present authors have observed that, when a trace of the acetates of an alkali or alkaline-earth metal is added to an alcoholic

* Cf. Decker and Fellenberg, 'Ann.,' vol. 364, p. 1 (1909).

solution of (I) or (II), the colour is immediately discharged, due to the pseudo-base formation. This clearly indicates that the acetates of these metals have not the power of forming stable addition complexes with the oxonium group. In the absence of any phenolic hydroxyls, no second colour change takes place. In contrast to the above, when sodium acetate is added to an alcoholic solution of cyanin chloride, the first colour change (red to purple) due to colour base formation, is followed by a second colour change (purple to blue) due to phenolic salt formation. The blue phenolic salt thus produced decolorises when allowed to stand in dilute solution.

The above observations have brought the authors to the conclusion that, in the fine blue complex iron salts of anthocyan-*ins*, the blue colour itself is due to an interaction of the metal with a phenolic group (or groups), whilst the stability of the colour in dilute solution is due to the attachment of the metallic salt to the oxonium complex. In the case of the blue alkali, or alkaline-earth salts of anthocyan-*ins*, the blue colour is due to a phenolic grouping, whilst the lack of stability in dilute solution is due to the inability of these salts to form stable complexes with the oxonium group.

The authors consider that it is most probable that all the anthocyan pigments form additive salts with ferric chloride, in which the iron salt is attached to the oxonium group, but that characteristic colours are only produced when suitably placed phenolic OH groups also react.

Conclusions concerning the Constitution of Blue Anthocyan Pigments in Flowers.

From the above it will be seen that the blue colours of anthocyan-containing flowers may be due to the presence of either—

- (i) Anthocyan phenolates of alkali or alkaline-earth metals; or
- (ii) Complex anthocyan-iron salts. (It is unlikely that copper or tin will take part.)

In deciding under which of these heads we must classify the actual blue pigments occurring in flowers, observations as to whether or no dilute solutions of their colouring matters become decolorised on standing should serve as a reliable guide.

In the case of at least three blue flowers (cornflower, iris, and violet), there is definite recorded evidence* that solutions of the blue pigments present in each of these flowers decolorise on standing. As there is also record that the decolorisation in each case is due to pseudo-base formation, and not to decomposition, the conclusion must be drawn that no complex

* Fremy and Cloez, 'J. f. pr. Chem.,' vol. 62, p. 269 (1854); Willstätter and Everest 'Ann.,' vol. 401, p. 189 (1913).

anthocyan-iron salt is present in these flowers. The blue colour must therefore be due to the presence of anthocyan phenolates of the alkali or alkaline-earth metals.

In the case of the flowers of the hydrangea, in which the pigment assumes a blue colour after the addition of iron salts to the soil in which the plant is grown, it is quite possible that we have an example of the other type, and that the pigment is a complex anthocyan-iron salt. As the authors have not yet been able to examine these flowers, a decision upon this point must be left until later.

The Mode of Formation of Anthocyan Pigments in Plants.

The extended botanical investigations of Keeble, Armstrong and Jones,* and of Wheldale,† indicated that a relationship existed between the yellow sap-pigments and the anthocyan, whilst Everest has described work which brought him to the conclusion that in Nature the anthocyan colouring matters are produced from yellow sap pigments (of the flavonol group) by reduction.‡

The present authors now describe observations which appear to prove that, at least in some cases, this conclusion is fully justified. The experiments now described were undertaken with a view to extending our insight into the mechanism of production of the anthocyan pigments in Nature.

Although it has already been established by the work of Willstätter and of Everest, that the anthocyan pigments may be produced by reduction of flavonol derivatives, it has not previously been so clearly indicated whether, in Nature, flavonols are first formed and from them the anthocyan, or the anthocyan directly synthesised and the flavonols produced from them by oxidation.

Additional interest was given to the matter by a lecture by Dr. R. Robinson, F.R.S. (then Professor of Chemistry in the University of Liverpool), at the University of Manchester, in which he dealt with the theoretically possible synthesis of plant products from sugars. He indicated the manner in which the synthesis of anthocyan from sugars *might* take place in Nature, and also pointed out that it was less easy to indicate how flavonols could be synthesised from sugars. As a result of these theoretical considerations, Dr. Robinson contended that the flavonols were formed from anthocyan by oxidation, the anthocyan being the primary synthetic products.

* 'Roy. Soc. Proc.,' B, vol. 85, p. 214 (1912); vol. 86, pp. 308 and 318 (1913); vol. 87, p. 113 (1913); and Keeble and Armstrong, 'Jour. Genetics,' vol. 2, p. 277 (1913).

† 'Camb. Phil. Soc. Proc.,' vol. 15, p. 137 (1909); 'Jour. Genetics,' vol. 1, p. 133 (1911); 'Biochem. Jour.,' vol. 7, p. 87 (1913).

‡ 'Roy. Soc. Proc.,' B, vol. 87, p. 444; vol. 88, p. 326; and vol. 90, p. 251.

The present paper describes an attempt made by the authors to indicate which of these possible processes of synthesis is in reality adopted by Nature.

If Robinson's suggestions are correct, then in those flowers which contain anthocyan pigments, it would be anticipated that the colour of the anthocyan would develop before any trace of flavonol could be detected. No published observations are yet available to support this, and the present results indicate that the opposite is the case. Further, even pale yellow, or white, flowers which contain flavonol derivatives but no anthocyan, would be expected to pass through a stage in which they were coloured by the anthocyan from which the flavonols were to be formed; unless it is assumed that the transformation from anthocyan to flavonol, in such cases, is always complete, and proceeds more rapidly than the formation of anthocyan.

If on the other hand flavonols are first synthesized in Nature and anthocyan formed from them only in those cases where the conditions for reduction are available, then the stages would be simplified. In the pale yellow or white petals containing flavonols there would be gradual development without marked colour changes. This is what occurs. In the flowers which are coloured by anthocyan it may well be expected that at least in most cases the very small buds will be pale yellow, or colourless, and that a certain amount of flavonol will be developed before the anthocyan is formed and becomes obvious by its tints. The present investigation proves that this is the case. Moreover, it is likely that flavonols would be found alongside anthocyan in flowers containing the latter. Published observations agree with this.

The authors consider that the present results strengthen the evidence that the anthocyan pigments are formed from flavonols. Only by assumptions such as that indicated above, could the reverse be reconciled with the experimental evidence at present available.

Experimental.

As myricetin^{*} was not available to the authors, and Shibata, Shibata and Kasiwagi state in their paper that the reduction of quercetin or of quercitrin proceeds "just the same as myricetin or myricitrin," the authors carried out a reduction of quercitrin under exactly the conditions given in the paper of the above mentioned authors. The result was that a green solution having bright green fluorescence^{*} was obtained from which a green pigment (mixture of pigments) could be precipitated by means of ether. The green precipitate remained green on washing with ether. An exactly similar result was obtained if the reduction was carried out by means of a very small amount of

hydrochloric acid, using excess of magnesium, and when the reduction was completed, adding magnesium acetate.

In order to show that the pigments obtained by the above-mentioned authors were not pure products, experiments were then made as follows. Chemically pure crystalline samples of cyanin chloride, violanin chloride, and quercitrin were used.

(i) Magnesium acetate was added to a fresh alcoholic-solution of (*a*) cyanin chloride; (*b*) violanin chloride. In each case a fine pure blue pigment was formed, which did not change when precipitated and washed with ether.

(ii) Magnesium acetate was added to a solution of quercitrin in alcohol; a bright yellow colour developed and yellow-green fluorescence. Ether precipitated a yellow pigment (Mg salt), which did not change when washed with ether.

(iii) Magnesium acetate was added to solutions containing (*a*) a mixture of cyanin chloride and quercitrin, and (*b*) a mixture of violanin chloride and quercitrin. In each case green pigments were at once produced, and the solutions showed the same bright green fluorescence as when the reduction above described was carried out. Precipitation and washing with ether left the green pigments (mixtures) unchanged.

(iv) An alcoholic solution of magnesium acetate was made, filtered, and ether added;—magnesium acetate was precipitated.

These results prove that the analyses of the green pigments described by the above mentioned authors are valueless.

By adjustment of the proportions of cyanin chloride and quercitrin in Experiment iii (*a*), the green pigment obtained by reduction of quercitrin was readily matched.

Further observations respecting the reaction of anthocyan pigments with metallic salts, which have been made from time to time, appear to be of sufficient interest to insert here. In particular, those on violanin, as this would most nearly correspond to the reduction products obtained from myricetin or myricitrin.

(i) To separate portions of a fresh solution of violanin chloride in alcohol (*circa* 95 per cent.) there were added: (*a*) magnesium acetate, (*b*) calcium acetate, (*c*) anhydrous sodium acetate; in each case a fine pure blue was at once produced, and on standing, the whole of the colour was gradually deposited as an indigo-blue flocculent precipitate. The colour of the blue solutions could not be distinguished from one another. The blue precipitates were all soluble in water, but were insoluble in absolute or 95 per cent. alcohol. The dilute solutions of these precipitates become decolorised on standing, owing to pseudo-base formation.

(ii) Effect of metallic salts on aqueous solutions of violanin chloride :—

(a) Addition of magnesium acetate gave a fine blue (with violanin mixed with quercitrin, green).

(b) Addition of ferrous sulphate gave a fine blue (with violanin mixed with quercitrin, green).

(c) Addition of copper sulphate produced a fine purple-blue, which passed to pure blue on warming, returning to purple-blue on cooling.

(iii) Cyanide chloride when in alcoholic solution behaved exactly like violanin chloride when magnesium acetate, anhydrous sodium acetate, or potassium acetate was added, a fine blue colour, followed by deposition of the blue pigment, being the result in each case.

(iv) Further observations that have been made by the present authors, and which have direct bearing upon the paper under discussion, are those upon the effect of adding various metallic salts to solutions of the colourless form of violanin. A solution was prepared by dissolving crystalline violanin chloride in a little alcohol, pouring this into water, and allowing to stand until decolorised. Separate portions of the solution were taken and salts added, with the results given below. (In each case one drop of a dilute solution of the salt was used.)

(a) Ferrous Ammonium Sulphate: no immediate colour, but fine blue gradually appeared.

(b) CuSO_4 : no immediate colour, but fine blue gradually appeared.

(c) SnCl_2 : no immediate colour, but fine blue gradually appeared.

(d) FeCl_3 : great difficulty in obtaining colour as oxidation with destruction of pigment occurred; if very dilute solution of FeCl_3 was used, got fair but pale blue.

(e) CaCl_2 : at once very pale green but colour did not increase.

(f) Magnesium Acetate: at once very pale green but colour did not increase.

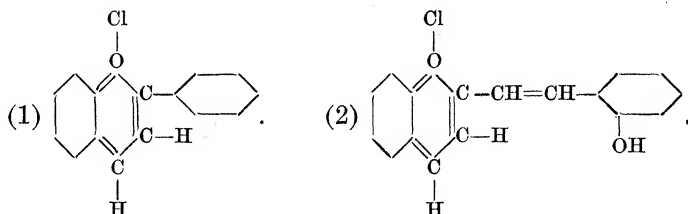
(g) Lead acetate: at once very pale green but colour did not increase.

(h) Sodium Acetate: at once very pale green but colour did not increase.

(i) Na_2CO_3 : rapidly yellow green; on standing, yellow and pigment destroyed.

Observations concerning Related Pyrylium Compounds.

In connection with other work, the present authors have prepared the mother substance from which all anthocyanins are derived (1), and also the related compound (2):—



The compound (1) has been prepared as the oxonium chloride, as a double salt with ferric chloride, and as the free base, whilst compound (2) has been isolated as oxonium chloride, and as a double salt with ferric chloride.

Observations on the Colour of Buds and Young Flowers.

A considerable number of plants, including auricula, apple, azalea, polyanthus (red), primrose (red), violas, etc., were selected whose flowers had well-marked anthocyan coloured petals when fully developed. Buds were taken in each case from the same plant at intervals and examined, until the fully developed flower had been reached. In some cases the anthocyan did not develop until light fell on the petals, in others the anthocyan (even quite deep shades) developed freely before the bud was unfolded. Despite this, in nearly every case it was evident that, before the anthocyan colour appeared, there was a stage in which the petals were yellow or colourless, and contained dissolved substances which turned yellow on exposure to ammonia. In the cases of some very deep coloured flowers, *e.g.*, black violas, the buds were highly coloured, even when extremely small.

A number of flowers which when fully developed contain only flavonols were likewise examined, including primrose (pale yellow), roses (various), chrysanthemum, etc., but in no case could any intermediate anthocyan formation be observed.

Attempts to Extract Flavonols from Buds (before Anthocyanins are present) and Produce Anthocyan Colorations from them.

Whilst the above observations showed that it was probable that flavonols were formed before anthocyanins, the authors desired to obtain further proof of the presence of the flavonols. To this end they chose buds of a red rose, and of a medium coloured mauve viola (Maggie Mott), as being

suitable on account of the fact that they attained a fair size before any sign of anthocyan formation became observable. Buds of these were gathered of the largest size attainable before any sign of anthocyan formation had appeared. The young petals were well crushed in warm alcohol (*circa* 95 per cent.), and, after standing for a short time, the extract was filtered, and the filtrate acidified by the addition of a small amount of concentrated hydrochloric acid. When the acid was added, the filtrate changed slightly from pale yellow to colourless (on the other hand, ammonia gave a deeper yellow), but in neither case was any red tint due to anthocyan observed. The acidified filtrate was divided into two portions; to one a little magnesium was added, the second was retained as a check. In the case of both rose and viola, a gradual change to a clear pale red took place in the colour of the liquid to which the magnesium had been added. The portion of the acidified extract which had not had magnesium added to it remained colourless. These observations leave no doubt that, in the cases examined, there were flavonol derivatives present in the buds which would later have developed anthocyan colours, but in which up to that time no anthocyan had been formed.

The authors intend to extend these observations.

A Remarkable Flint Implement from Selsey Bill.

By Sir RAY LANKESTER, K.C.B., F.R.S.

(Received January 18, 1921.)

[PLATES 8-11.]

It is desirable that the large rostrate flint implement drawn in figs. 1, 2, and 3, and the hammer-stones drawn in figs. 4 and 5 should be made known without further delay to students of prehistoric archaeology. These specimens were placed at my disposal in 1912 by Mr. Edward Heron Allen, F.R.S., and have now been presented to the Department of Ethnology and Mediæval Antiquities of the British Museum. They were briefly mentioned by me in a postscript to my paper on "The Discovery of a novel type of Flint Implements below the base of the Red Crag of Suffolk."*

These specimens were (as I am informed by Mr. Heron Allen) found in November, 1911, when the shingle was suddenly washed away below the

* 'Phil. Trans.,' B, vol. 202. p. 332.